Impact of genetic variations in the MAPK signaling pathway on outcome in metastatic colorectal cancer patients treated with first-line FOLFIRI and bevacizumab: data from FIRE-3 and TRIBE trials

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Background: The MAPK-interacting kinase 1 (MKNK1) is localized downstream of the RAS/RAF/ERK and the MAP3K1/MKK/p38 signaling pathway. Through phosphorylation MKNK1 regulates the function of eukaryotic translation initiation factor 4E, a key player in translational control, whose expression is often upregulated in metastatic colorectal cancer patients (mCRC). Preclinical data suggest that MKNK1 increases angiogenesis by upregulating angiogenic factors. We therefore hypothesize that variations in the MKNK1 gene predict outcome in mCRC patients treated with first-line FOLFIRI and bevacizumab (bev).

Patients and methods: A total of 567 patients with KRAS wild-type mCRC in the randomized phase III FIRE-3 and TRIBE trials treated with first-line FOLFIRI/bev (discovery and validation cohorts) or FOLFIRI and cetuximab (cet) (control cohort) were included in this study. Five single-nucleotide polymorphisms in the MAPK signaling pathway were analyzed.

Results: AA genotype carriers of the MKNK1 rs8602 single-nucleotide polymorphism treated with FOLFIRI/bev in the discovery cohort (FIRE-3) had a shorter progression-free survival (PFS) than those harboring any C (7.9 versus 10.3 months, Hazard ratio (HR) 1.73, P = 0.038). This association could be confirmed in the validation cohort (TRIBE) in multivariable analysis (PFS 9.0 versus 11.0 months, HR 3.04, P = 0.029). Furthermore, AA carriers in the validation cohort had a decreased overall response rate (25% versus 66%, P = 0.049). By combining both FOLFIRI/bev cohorts the worse outcome among AA carriers became more significant (PFS 9.0 versus 10.5 months) in univariable analysis (HR 1.74, P = 0.015) and multivariable analysis (HR 1.76, P = 0.022). Accordingly, AA carriers did also exhibit an inferior overall response rate compared with those harboring any C (36% versus 65%, P = 0.005).

Conclusion: MKNK1 polymorphism rs8602 might serve as a predictive marker in KRAS wild-type mCRC patients treated with FOLFIRI/bev in the first-line setting. Additionally, MKNK1 might be a promising target for drug development.

Key words: MKNK1, single-nucleotide polymorphisms, FOLFIRI/bevacizumab, metastatic colorectal cancer, predictive biomarker
Colon cancer is the fourth leading cause of cancer-related death worldwide [1]. In the last decade prognosis of metastatic colorectal cancer (mCRC) patients considerably improved mainly due to the introduction of biologics [2]. However, to further improve prognosis and overcome treatment resistance in refractory mCRC new treatment options are eagerly awaited. Preclinical in vitro and in vivo data suggest a critical role for the MAPK-interacting kinase 1 (MKNK1)-mediated eIF4E activation in tumor development and progression [3,4]. MKNK1 is a serine/threonine kinase localized downstream of the RAS/RAF/MEK/ERK and the MEKK1/MEK/p38 signaling pathways [5]. MKNK1 regulates diverse biologic processes including translation, cell proliferation, and differentiation through phosphorylation of different substrates such as the eukaryotic translation initiation factor 4E (eIF4E), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), and Sprouty2 (Spry2) [6–8]. MKNK1 itself is phosphorylated and activated by p38, MAPK, and ERK, which are localized upstream. Through phosphorylation MKNK1 regulates the function of eukaryotic translation initiation factor 4E (eIF4E), a key player in translational control, whose expression is mostly upregulated in cancer patients [9–11]. In colorectal cancer patients eIF4E expression was markedly elevated in tumor tissue compared with adjacent normal colonic epithelial tissue [12]. Increased eIF4E promotes translation of mRNA encoding for proteins involved in cell cycle regulation such as c-myc, cyclin D1, apoptosis (survivin), and angiogenesis [13]. By enhancing translation of these tumor-associated RNAs, upregulated eIF4E stimulates tumorigenesis [14]. Noteworthily, in MKNK1 knock-out mice normal cell development was not impaired, rendering this protein to a promising anticancer target [15]. Preclinical data also suggest that MKNK1 stimulates angiogenesis and endothelial cell migration by upregulating angiogenic factors [16,17], which led us to explore the impact of genetic variations in the MAPK signaling pathway, especially MKNK1 and its substrates on outcome in patients with mCRC treated with first-line FOLFIRI and bevacizumab (bev).

### Patients and methods

#### Study design and patient population

A total of 567 patients with KRAS exon 2 wild-type mCRC enrolled in the randomized phase III FIRE-3 and TRIBE trials and treated with either first-line FOLFIRI/bev (discovery and validation cohorts) or FOLFIRI and cetuximab (cet) (FIRE-3, control cohort) were included in this study. In FIRE-3 FOLFIRI/bev group bev was administered at a dose of 5 mg/kg every 2 weeks and in the FOLFIRI/cet cohort the first cet infusion was given at a dose of 400 mg/m2, thereafter 250 mg/m2 weekly. The FOLFIRI regimen was administered as follows: 180 mg/m2 irinotecan, 400 mg/m2 leucovorin, 400 mg/m2 fluorouracil (5-FU) bolus infusion and 2400 mg/m2 continuous infusion over 46 h. The treatment was repeated every 2 weeks until disease progression or unacceptable toxic side-effects developed [18]. The validation cohort comprised 94 KRAS exon 2 wild-type mCRC patients enrolled in the randomized phase III TRIBE trial and treated with the same regimen (FOLFIRI/bev) as described above. However, leucovorin was administered at a dose of 200 mg/m2. After 12 cycles, patients in TRIBE received a maintenance therapy with 5-FU/bev until disease progression [19].

The study was approved by the local ethics committees for each participating site. All patients provided informed consent for the analysis of molecular correlates. Molecular analyses were carried out at the USC/ Norris Comprehensive Cancer Center in Los Angeles. Our study was conducted adhering to the reporting recommendations for tumor marker prognostic studies [20].

#### Candidate polymorphisms

Potentially functional single-nucleotide polymorphisms (SNPs) within genes involved in the MKNK1 signaling pathway were identified according to the following criteria: minor allele frequency >10% in Caucasians; potential to change gene function in a relevant matter according to public databases (https://snpinfo.niehs.nih.gov, compbio.cs.queensu.ca, https://www.ncbi.nlm.nih.gov as well as www.genecards.org) 20 June 2017, date last accessed).

#### Genotyping

Genomic DNA extraction was carried out from formalin-fixed paraffin-embedded tissue in the discovery and control cohorts and from blood in the validation cohort using the QiAmp DNA easy kit (Qiagen, Valencia). Six functional SNPs in six genes (MKNK1, eIF4E, eIF4G1, 4EBP1, hnRNPA1, and Spry2) were analyzed by PCR-based direct sequencing. Forward and reverse primers (supplementary Table S1, available at Annals of Oncology online) were used for PCR amplification. PCR fragments were then sequenced on an ABI 3100A Capillary Genetic Analyzer (Applied Biosystem) to identify the SNP. The investigator (MDB) reading the sequence was blinded to the clinical outcome data.

#### Statistical analysis

The aim of this study was to identify SNPs within the MAPK signaling pathway and their associations with clinical outcome in mCRC patients enrolled in two phase III randomized trials, namely the FIRE-3 and TRIBE trials. The discovery cohort consisted of patients receiving first-line FOLFIRI/bev within the FIRE-3 trial, whereas patients treated with the same regimen in TRIBE served as a validation set. The control cohort consisted of patients receiving first-line FOLFIRI/cet in FIRE-3.

Primary endpoint was progression-free survival (PFS) and secondary endpoints were overall survival (OS) and overall tumor response rate (ORR). PFS was defined as time from randomization until disease progression, death or until last follow-up in patients who were alive and remained free of disease progression. OS was defined as time from randomization until death. Patients still alive were censored at the last date of follow-up. ORR represented the percentage of patients who achieved either a complete (CR) or a partial (PR) remission according to the Response Evaluation Criteria in Solid Tumors (RECIST). Allelic distribution of genetic variants was tested for deviation from Hardy–Weinberg equilibrium (HWE) using the \( \chi^2 \) test. Differences between baseline characteristics among the three cohorts were compared by using the \( \chi^2 \) test. To evaluate the effects of different SNPs on PFS and OS log-rank test was used in the univariable analysis and Wald test in the Cox proportional hazards model adjusting for patient characteristics that remained significantly associated with clinical outcome in the multivariable analysis \( (P < 0.10) \). The adjusting factors for multivariate analyses in each cohort are described in Table 1 and supplementary Table S3, available at Annals of Oncology online. The associations between SNPs and tumor response were evaluated using the Fisher’s exact test and a multivariable logistic regression model adjusting for ECOG performance status, previous adjuvant chemotheraphy, and BRAF status. Significant SNPs associated with clinical outcome in the discovery cohort (FIRE-3) were tested in the validation set (TRIBE) and in the control cohort (FIRE-3).

With 247 patients (205 PFS events) in the FIRE-3 FOLFIRI/bev arm with specimen available for genotyping, we would have 80% power to...
Table 1. Association between MKNK1 rs8602 with clinical outcomes among KRAS exon 2 wild-type patients

<table>
<thead>
<tr>
<th>Tumor response</th>
<th>Progression-free survival</th>
<th>Overall survival</th>
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<tr>
<td></td>
<td>N</td>
<td>PR + CR</td>
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<td>(95%)</td>
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**Discovery cohort**

| C/C | 124 | 73 (65%) | 40 (35%) | 0.17 | 0.12 | 0.10 | 0.15 | 26.1 (15.3, 30.3) | 1 (Reference) | 1 (Reference) |
| C/A | 93  | 55 (65%) | 30 (35%) | 0.068 | 0.039 | 0.038 | 0.0056 | 25.4 (13.0, 28.1) | 1 (Reference) | 1 (Reference) |
| A/A | 19  | 7 (41%)  | 12 (65%) | 0.0089 | 0.071 | 0.34 | 0.077 | 18.4 (9.8, 29.0) | 1 (Reference) | 1 (Reference) |

Any C: 217 128 (65%) 70 (35%) 0.049 0.021 0.17 0.029 28.8 (22.0, 36.1) 1 (Reference) 1 (Reference)

**Validation cohort**

| C/C | 50  | 32 (65%) | 17 (35%) | 0.019 0.008 0.051 0.072 | 0.35 0.21 |
| C/A | 36  | 23 (68%) | 11 (32%) | 0.05 0.02 |
| A/A | 8   | 12 (25%) | 6 (75%)  | 0.005 0.002 0.015 0.022 |

Any C: 86 55 (65%) 28 (34%) 0.019 0.008 0.051 0.0072 0.35 0.21 |

**Combined cohort**

| C/C | 174 | 105 (65%) | 57 (35%) | 0.005 0.002 0.015 0.022 |
| C/A | 129 | 78 (69%) | 41 (34%) | 0.005 0.002 0.015 0.022 |
| A/A | 27  | 9 (36%)  | 16 (64%) | 0.005 0.002 0.015 0.022 |

**Control cohort**

| C/C | 136 | 88 (72%) | 34 (28%) | 0.09 0.01 0.48 0.65 |
| C/A | 76  | 44 (71%) | 18 (29%) | 0.09 0.01 0.48 0.65 |
| A/A | 14  | 8 (80%)  | 2 (20%)  | 0.09 0.01 0.48 0.65 |

Any C: 212 132 (72%) 52 (28%) 0.73 0.53 0.33 0.066 0.076 0.26 |

Any A: 14 8 (80%) 2 (20%) 0.73 0.53 0.33 0.066 0.076 0.26 |

**P** value is based on Fisher’s exact test for tumor response, log-rank test for PFS and OS in the univariable analysis (†), and Wald test in the multivariable Cox proportional hazards regression model (‡) adjusting for age, ECOG performance status, primary tumor site, liver limited metastasis, primary tumor resection, adjuvant chemotherapy, and BRAF status in the discovery, control and combined cohorts; adjusting for sex, age, ECOG performance status, primary tumor site, liver limited metastasis, primary tumor resection, adjuvant chemotherapy, and BRAF status in the validation cohort.

**P** value was based on multivariable logistic regression model adjusting for ECOG performance status, adjuvant chemotherapy, and BRAF status.

PR, partial remission; CR, complete remission; SD, stable disease; PD, progressive disease. Significant **P** values are in bold.

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detect a minimum hazard ratio (HR) of 1.48–1.66 for a SNP with minor allele frequency of 0.1–0.5 on PFS using a two-sided 0.05 level log-rank test. The HR of 1.51–1.70 would be detected in the FIRE-3 FOLFIRI/cet arm with 226 patients (189 PFS events) and 1.97–2.36 in the FOLFIRI/bev arm of TRIBE with 94 patients (69 PFS events) for the same SNP using the same test and assuming the same power and allele frequencies. All P-values were from two-sided Wald tests at a 0.05 significance level. All tests were carried out by using the SAS statistical package version 9.4 (SAS Institute, Cary).

Results

Baseline characteristics of the three cohorts (discovery, validation, and control sets) are depicted in supplementary Table S2, available at Annals of Oncology online. Shortly, the study comprised 567 KRAS exon 2 wild-type mCRC patients in total, 247 in the discovery cohort (FIRE-3 FOLFIRI/bev arm), 226 in the control cohort (FIRE-3 FOLFIRI/cet arm), and 94 in the validation cohort (TRIBE FOLFIRI/bev arm). The association of all 5 SNPs with outcome in the discovery cohort is outlined in supplementary Table S3, available at Annals of Oncology online. The allelic distribution of all genetic variants examined were within the HWE in each cohort (P > 0.05), except for eIF4G1 rs2178403 SNP (P = 0.03). We therefore excluded this SNP from further analyses. The MKNK1 rs8602 SNP showed significant association with PFS in patients treated with first-line FOLFIRI/bev in FIRE-3 (discovery cohort). Here, AA genotype carriers had a markedly shorter median PFS than those harboring any C allele (7.9 versus 10.3 months) in univariate analysis [HR 1.73, 95% confidence interval (CI) 1.02–2.95, P = 0.038] (Table 1 and Figure 1A). The same trend in PFS could be shown in patients receiving FOLFIRI/bev in the validation cohort (TRIBE). Again, patients with the AA genotype showed a decreased PFS compared with those having any C allele (9.0 versus 11.0 months, HR 1.86, 95% CI 0.72–4.78, P = 0.17 in univariate analysis) (Figure 1B). However, in multivariable analysis this association became statistically significant (HR 3.04, 95% CI 1.12–8.27, P = 0.029). Consistent with the previous findings, AA carriers in both the discovery and validation cohorts exhibited a lower ORR compared with those having any C allele (41% versus 65%, P = 0.068 and 25% versus 66%, P = 0.049, respectively). Here again, these associations became more significant in multivariate analyses in both cohorts.
To the best of our knowledge, we provide the first evidence that variations in the MKNK1 gene may predict outcome in mCRC patients treated with first-line FOLFIRI/bev. While we could demonstrate that KRAS wild-type mCRC patients carrying any C allele (9.0 versus 10.5 months) in both univariable (HR 1.74, 95% CI 1.10–2.77, \( P = 0.015 \)) and multivariable analysis (HR 1.76, 95% CI 1.09–2.86, \( P = 0.022 \)). Similarly, mCRC patients with an AA genotype displayed a markedly impaired ORR compared with those harboring any C allele (36% versus 65%, \( P = 0.005 \) in univariate and \( P = 0.002 \) in multivariate analyses).

**Discussion**

To the best of our knowledge, we provide the first evidence that variations in the *MKNK1* gene may predict outcome in mCRC patients treated with first-line FOLFIRI/bev. While we could demonstrate that KRAS wild-type mCRC patients carrying any C allele have a better PFS than patients having any A allele (9.0 versus 10.5 months) in both univariable (HR 1.74, 95% CI 1.10–2.77, \( P = 0.015 \)) and multivariable analysis (HR 1.76, 95% CI 1.09–2.86, \( P = 0.022 \)). Similarly, mCRC patients with an AA genotype displayed a markedly impaired ORR compared with those harboring any C allele (36% versus 65%, \( P = 0.005 \) in univariate and \( P = 0.002 \) in multivariate analyses).

**elF4E in regulating VEGF** [16]. Similarly, in breast cancer patients *elF4E* overexpression in tumor samples was closely associated with increased expression of angiogenic factors such as *VEGF, IL-8, and FGF-2* and microvessel density [24–26]. Another study by Xu et al. demonstrated that colon cancer patients exhibiting higher *elF4E* protein levels have an enhanced risk to develop liver metastases suggesting that overexpression of *elF4E* results in increased angiogenesis and thereby facilitates tumor cell spreading [27]. Similarly, Nathan et al. could show that an increase in *elF4E* during head and neck tumorigenesis correlates with higher VEGF, *b-FGF* and microvessel density [28].

The influence of the *MKNK1-elF4E* axis on angiogenesis may explain why we observe differences in outcome only in patients treated with FOLFIRI/bev but not in those receiving FOLFIRI/cet. One might assume that *MKNK1* activation might circumvent VEGF blockade of bevacizumab by selectively activating alternative angiogenic factors to further promote angiogenesis. Therefore, targeting *MKNK1* may be a promising approach to enlarge our treatment armamentarium against mCRC and to overcome resistance in patients treated with bevacizumab based chemotherapy. Available data on *MKNK1* are almost limited to experimental models indicating its role in tumorigenesis [3, 15, 29] and preclinical studies showing activity of *MKNK1* inhibitors against different types of solid tumors [9, 14]. Due to these promising results, a clinical phase I–II trial evaluating the safety and activity of daily oral *MKNK1* inhibitor (eFT508) has been initiated and is currently recruiting patients with treatment refractory advanced solid tumors (NCT02605083, https://clinicaltrials.gov/ct2/show/NCT02605083 (20 June 2017, date last accessed)).

In our study, the significant association of the *MKNK1* SNP rs8602 with PFS in KRAS exon 2 wild-type mCRC patients treated with first-line FOLFIRI/bev in two independent phase III studies confirms our initial hypothesis and support furthermore previous findings from other groups suggesting an influence of *MKNK1* in upregulating angiogenesis [23–26].

Due to the limited number of patients with KRAS exon 2 mutations enrolled before the protocol amendment in FIRE-3 (discovery cohort), and the low frequency of *MKNK1* rs8602 AA genotype carriers, we did not analyze the association of this SNP on outcomes in KRAS exon 2 mutant patients, as it would not allow us to draw any firm conclusion. Similarly, sample size restrictions and the low prevalence of AA carriers precluded us from performing further subgroup analyses in *RAS* wild-type/mutant or *BRAF* mutant patients. Therefore, we intentionally restricted our analysis to KRAS exon 2 wild-type patients, who represented the intention-to-treat population in FIRE-3 (discovery cohort).

The strength of our study is that we included 567 KRAS wild-type mCRC patients enrolled in three different cohorts of two phase III randomized trials who were treated with either FOLFIRI/bev or FOLFIRI/cet. Secondly, our findings obtained in the discovery cohort could be confirmed in the validation cohort. A limitation of our study is the small number of patients harboring any AA genotype. However, by combining both cohorts of FIRE-3 and TRIBE FOLFIRI/bev arms the decreased PFS among AA carriers became more evident.

In conclusion, our results suggest that the *MKNK1* polymorphism rs8602 might serve as a predictive marker in KRAS wild-type patients with mCRC treated with FOLFIRI/bev in the first-line setting.
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Disclosure

The authors have declared no conflicts of interest.

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